Prevalence, Distribution, and Antimicrobial Susceptibility of *Staphylococcus aureus* in Ready-to-Eat Salads and in the Environment of a Salad Manufacturing Plant in Northern Greece

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Abstract

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The prevalence, distribution, and antibiotic susceptibility of *Staphylococcus aureus* was investigated in ready-to-eat (RTE) salads, the environment, and the personnel of a salad producing plant in Greece. Staphylococci were isolated from 27% of the samples. Apart from three isolates which were sensitive to all antibiotics, all the others exhibited resistance from two up to five antibiotics. None of the isolates was resistant to oxacillin. Random Amplified Polymorphic DNA (RAPD) analysis showed no genetic relation between the human and salad isolates while one RAPD clone of *S. aureus* predominated among the salad samples. The results suggest that an effective application of Good Manufacturing Practices (GMP) is needed along the food production chain to prevent the contamination of RTE foods.

Keywords: Staphylococci; antimicrobial susceptibility; RTE salads; environment

Staphylococci are common inhabitants of healthy humans and domestic and food animals, and they are isolated from several sites of their bodies, including the nose, throat, skin, hairs, and stool (Martin & Myers 1994; Sears & McCarthy 2003; Weese 2005; Hanselmann *et al.* 2006). Staphylococci may also exist in food products of animal origin or those that are handled directly by humans (Jay *et al.* 2005). *Staphylococcus aureus* gastroenteritis is one of the most prevalent foodborne bacterial intoxications worldwide (Jablonski & Bohach 2001; Stewart 2005). Furthermore, staphylococci resistant to beta-lactam antibiotics are among the prominent nosocomial pathogens (Lim & Webb 2005). Antimicrobial resistance represents a global important public health concern (Harrison & Lederberg 1998; Finch & Hunter 2006). The increasing antibiotic resistance of nosocomial isolates of *S. aureus* poses a great problem for the management of the hospital acquired infections (Lauria & Angeletti 2003; Goosens 2005). Although traditionally the acquisition of methicillin resistant *Staphylococcus aureus* (MRSA) has been considered restricted

to the hospital settings, the community acquired infections caused by MRSA have emerged as an important public health issue over the last decade (Maltezou & Giamarellou 2006).

The distribution of MRSA in the environment is of great concern for human infections and eating or handling contaminated foods may be a potential route of transmission according to the European Food Safety Authority (EFSA 2009). Specific studies are needed to examine the incidence of MRSA in foods and the environments of food producing facilities in order to evaluate the relative risk of foods as sources of infection in humans. So far, MRSA have been isolated from food producing animals (Vanderhaegen *et al*. 2010) and foods of animal origin, such as meat products from cattle and pigs (van Loo *et al*. 2007), milk and dairy products (Normanno *et al*. 2007). MRSA has been associated with food both through contamination from humans (Kluytmans *et al*. 1995) and due to the colonisation of food-producing animals (Voedsel en Waren Autoriteit 2007). Food sampling and examination should be focused on foods of animal origin and especially on RTE foods whose processing involves significant handling and which are consumed without any prior decontamination practice.

The aim of the present study was to investigate the occurrence, distribution, antimicrobial susceptibility, and genetic relatedness of *Staphylococcus aureus* isolates from RTE salads, their basic ingredients, the environment and the personnel of a salad manufacturing plant in Northern Greece. A Hazard Analysis and Critical Control Point (HACCP) system in the production control was applied in the plant.

MATERIAL AND METHODS

Samples. Four types of salad (cheese, fish roe, egg plant, and tzatziki) made in a salad manufacturing plant in Northern Greece were examined. The ingredients for the cheese salad were: mytzithra (whey cheese), yogurt, soybean oil, and hot chili pepper; for the tzatziki salad: yogurt, cucumber, and garlic; for the egg plant salad: roasted egg plant, mayonnaise, garlic, spices and vinegar; and for the fish roe salad: the paste of salted fish roe, bread, and soybean oil. A total of 80 salad samples of the 4 salads (20 samples for each salad type) and 50 samples of 5 basic salad ingredients of the salads (10 samples each of feta cheese, mytzithra, mayonnaise, fish roe, and roasted egg pulp) were examined. In addition, 80 swab samples from the environment (40 taken during the production and 40 taken 1 h after the standard sanitation procedures), 12 samples from workers' nasal cavities, 8 samples from the gloves, and 10 samples from the coats of the workers were also examined. The surfaces of the walls, floors, drainage lids, refrigerator knobs, mixer tanks and accessories, pulping machines, working tables, filling spoons, reusable plastic containers, plastic frames and tanks of the plant were also investigated for the presence of staphylococci. The surface swabs were always taken from the same sites. The samples from the nasal cavities of the personnel and the surfaces of their gloves and coats were taken during the salads production. Sampling took place in four trips (2 months apart) over a six months period.

The samples (250 g) of the salads and their basic ingredients were aseptically obtained, placed into stomacher bags, transferred to the laboratory under refrigeration conditions at 4°C, and processed as described below within 3 h from the collection. Workers' hands and plant surfaces (100 cm^2) were swabbed twice by the wet-dry double swab technique using sterile cotton swabs moistened with 0.1% peptone water containing 0.85% NaCl, followed by a second swabbing using a dry swab. The two swabs were pooled in one sample into tubes containing 10 ml of Tryptone Soy Broth (TSB; Biokar Diagnostics, Pantin, France) with 7.5% NaCl. The samples from personnel's nasal cavities were taken by swabbing both nostrils of each person using one sterile cotton swab per nostril. The swabs were previously moistened in the same medium as the swabs for surface sampling. The two swabs were pooled in one sample into tubes containing 10 ml TSB with 7.5% NaCl.

Isolation, enumeration, and identification of S. aureus. The isolation and enumeration of staphylococci was carried out as described previously (Abrahim *et al*. 2010). The enumeration of staphylococci was performed only in salads and their basic ingredients. Portions of 25 g taken from the samples were placed into stomacher bags containing 225 ml TSB with 7.5% NaCl. They were homogenised for 2 min in a stomacher (Lab Blender 400; A.J. Seward and Co. Ltd., London, UK) and then 10-fold serial dilutions were prepared in the same broth. One ml from each dilution was plated using pour plating technique onto Baird Parker agar supplemented with rabbit plasma

fibrinogen (bioMérieux, Marcy l'Etoile, France). For the detection of less than 10 CFU/g, the first dilution was incubated for enrichment at 37°C for 24 hours. One loopful of the enriched culture was spread plated onto the same agar and incubated at 37°C for 24–48 hours. The swabs were incubated overnight at 37°C in TSB broth with 7.5% NaCl for enrichment, and then a loopful of each broth was also plated onto Baird-Parker agar with rabbit plasma fibrinogen (detection limit < 1 log CFU/g) and the plates were incubated aerobically at 3°C for 24–48 hours.

After the incubation, three colonies typical of *S. aureus* were selected from each plate and transferred onto Tryptone Yeast Extract agar (TSEY; LAB M, Lancashire, UK) for purification and further identification. Preliminary identification was based upon Gram staining, positive catalase reaction, morphological and cultural characteristics. The isolates were identified at the species level on the basis of biochemical characterisation by the semi-automated system WIDER (Francisco Soria Melguizo, Madrid, Spain) using the Gram positive minimal inhibitory concentration/identification (MIC/ID) panels and API Staph System (bioMérieux, Craponne, France).

Antibiotic susceptibility. All isolates were tested for antibacterial susceptibility to 20 antibiotics often used in Greek hospitals. Minimum inhibitory concentration (MIC) was evaluated according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2006) in the semi-automated system WIDER (Francisco Soria Melguizo, Madrid, Spain) using the Gram Positive MIC/ID Panels. These antibiotics were: beta-lactams (penicillin – P, ampicillin – AMP, oxacillin – OX and amoxicillin/clavulanic acid – A/C), cephalosporins (cefazolin – CFZ and cefotaxime – CFT), aminoglycosides (streptomycin 1000 –S, gentamicin – G, gentamicin 500 – G500 and amikacin – AMC), glycopeptides (vancomycin – VAN and teicoplanin – TEI), fluoroquinolones (levofloxacin – LEV), macrolides (erythromycin – E), linkosamides (clindamycin – CL), streptogramins (quinupristin/dalfopristin – Q/D), oxazolidinones (linezolid – LIN), rifamycins (rifampin – RIF), chloramphenicol – CHL, fosfomycin – FOS and trimethoprim/sulfamethoxazole – T/S.

Random Amplified Polymorphic DNA fingerprinting. The isolates were studied by RAPD fingerprinting using the random arbitrary primer BG2 according to van Belkum *et al*. (1993).

RESULTS AND DISCUSSION

Staphylococcus aureus was isolated from 66/244 (27%) of the samples (Table 1). After enrichment staphylococci were recovered from 25 of the 48 positive samples from salads and their basic ingredients. The counts in the other 23 samples ranged from 2 × 10^2 CFU/g to 5 × 10^4 CFU/g (mean value 1.5 × 10^3 CFU/g, standard deviation 0.678). The rates of isolation in the salads and their basic ingredients ranged from 15% for tzatziki up to 60% for fish roe. The rates of *S. aureus* isolation from personnel's nasal cavities and their coats and gloves were 50%, 60%, and 50%, respectively. *S. aureus* was not detected in any of the environmental samples.

MRSA was not detected in any of the samples. All isolates were methicillin sensitive (MSSA), even those taken from workers' nasal cavities, although two of them had been hospitalised a few weeks

Table 1. Prevalence of *Staphylococcus aureus* from RTE salads, the environment and the personnel of a salad manufacturing plant

Sample origin	Samples (*n*)	Samples positive for *S. aureus* (%)
Salads		
Cheese salads	20	9 (45)
Roe salads	20	6 (30)
Tzatziki	20	3 (15)
Egg plant salads	20	9 (45)
Basic ingredients		
Roasted egg plant pulp	10	4 (40)
Mayonnaise	10	3 (30)
Feta cheese	10	4 (40)
Myzithra cheese	10	4 (40)
Fish roe	10	6 (60)
Food contact surfaces		
During production	25	
After sanitation	25	
Non food contact surfaces		
During production	15	
After sanitation	15	
Personnel		
Gloves	12	6 (50)
Coats	10	6 (60)
Nasal cavities	12	6 (50)
Total	244	66 (27)

ago and other two had been often visiting their relatives in intensive care units. *S. aureus* isolates exhibited resistance to at least two antibiotics with the exception of three isolates which were sensitive to all antibiotics (Table 2). A high rate of resistance was seen against ampicillin (94%), erythromycin (89.4%), fosfomycin (82.6%), and penicillin (59.1%) (Table 3). The most common resistance pattern was AMP-E-FOS-P (32 isolates) followed by AMP-E-FOS (18 isolates) (Table 2).

S. aureus isolates from feta cheese, myzithra cheese and cheese salad showed an identical antimicrobial pattern. This may be explained by the fact that they were produced by the same dairy plant. Identical antimicrobial pattern was also observed among the isolates from personnel's gloves and coats and those from mayonnaise (Table 2).

Human *S. aureus* isolates exhibited an antimicrobial profile different from those of the other isolates. Similar results were taken by RAPD, as the patterns of the human isolates were different from those isolated from salads and basic ingredients, suggesting that there was no transmission from humans to the products and vice versa (Figure 1). RAPD patterns obtained from all human isolates as well as from representative isolates from the salads are seen in Figure 1. Although the patterns from humans differed from those of the salads, two groups of *S. aureus* isolates (2 isolates each) were seen: isolates 1 and 3, and 4 and 5 were identical. One *S. aureus* RAPD profile was the most common; it was seen among all four kinds of salads. An example is seen in Figure 1, in which the patterns of isolates 7, 8, 9, 10, 11, and 12 are identical; isolate 7 was recovered from tzatziki salad, isolates 8, 9, 11, 12 from cheese salad, and isolate 10 was recovered from fish roe salad, suggesting that there was a common source of contamination.

Table 2. Antibiotic resistance patterns for *Staphylococcus aureus* isolated from RTE salads, the environment and the personnel of a salad manufacturing plant

Staphylococcus spp.	Resistance pattern*	Strains (*n*)	Sample origin
S. aureus	AMP, E, FOS, P	9	cheese salad
		4	roe salad
		2	tzatziki
		4	feta cheese
		4	mytzithra cheese
		3	fish roe
		6	Personnel's coats
	AMP, E, FOS	9	egg plant salad
		3	mayonnaise
		6	personnel's gloves
	AMP, E, G 500, FOS, P	2	roe salad
		1	roe egg pulp
	AMP, E, FOS, P, RIF	1	tzatziki
	AMP, E, FOS, T/S	4	roasted egg plant pulp
	AMP, P	2	personnel's nasal cavities
	AMP, E, P	1	prerssonnel's nasal cavities
	AMP, CHL, LEV(I), P	1	personnel's nasal cavities
	CHL, CL, Q/D, T/S	1	personnel's nasal cavities
	sensitive to all**	1	personnel's nasal cavities
		2	fish roe

*amoxicillin/clavulanic acid (A/C), amikacin (AMC), ampicillin (AMP), cefotaxime (CFT), cefazolin (CFZ), chloramphenicol (CHL), clindamycin (CL), erythromycin (E), fosfomycin (FOS), gentamicin (G), gentamicin 500 (G500), levofloxacin (LEV), oxacillin (OX), penicillin (P), quinupristin/dalfopristin (Q/D), rifampin (RIF) and trimethoprim/sulfomathoxasole (T/S); **intermediate resistance

Table 3. Resistance percentage to antimicrobials of *S. aureus* strains isolated from RTE salads, the environment and the personnel of a salad manufacturing plant

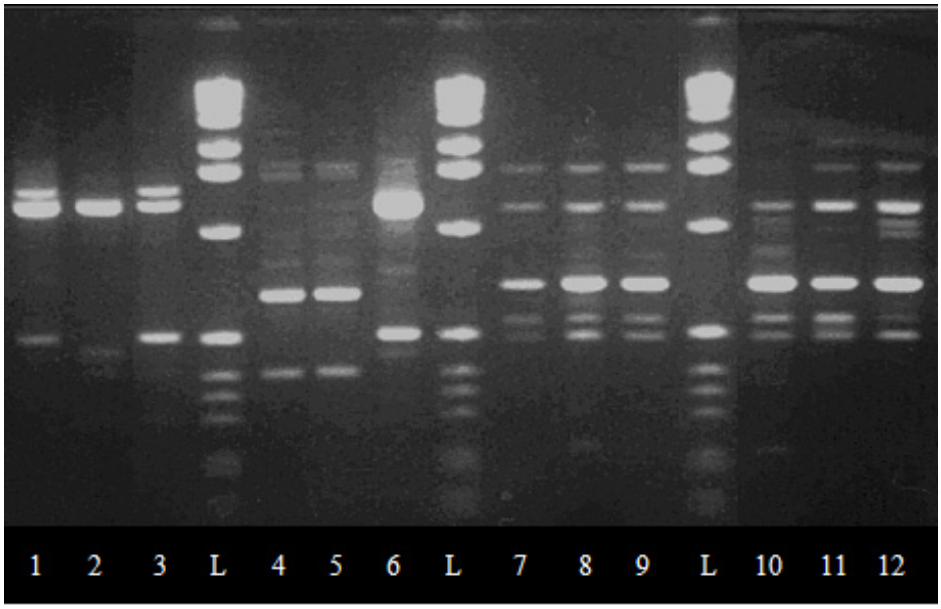
Antibiotics	*S. aureus* (%)
Ampicillin	94
Chloramphenicol	3
Clindanycin	1.5
Erythromycin	89.4
Fosfomycin	82.6
Gentamicin 500	4.5
Penicillin	59.1
Quinupristin/Dalfopristin	1.5
Rifampin	1.5
Trimethprimm/sulfomethoxasole	1.5

Although *S. aureus* is routinely enumerated in a variety of RTE foods as part of general microbiological safety checks in Europe (Anonymous 2005), there is a lack of information relating to antibiotic susceptibility of the isolated strains. According to other studies (Normanno *et al.* 2007; de Boer *et al.* 2009; Lozano *et al.* 2009), the majority of the examined strains of MRSA were isolated from raw foods which, if properly processed and prepared before consumption, is not likely to cause disease. However, there is generally a shortage of data concerning the presence of MRSA in RTE foods. Similarly to our survey, in Japan 315 japanese and western style desserts and 247 human hands were negative for MRSA (Shimamura *et al.* 2006). There is also a lack of studies on the prevalence of MRSA in the environment of food production plants. So far, it has been reported that staphylococci may become endemic in the environment of abattoirs (Borch *et al.* 1996), and several reports suggest that human *S. aureus* may become part of the endemic flora of food handlers, with subsequent contamination of carcasses and meat (Vanderlinde *et al.* 1999; Desmarchelier *et al.* 1999; Schlegelová 2004). In the study performed in the Czech Republic, *S. aureus* and *S. epidermidis* were isolated from food contact surfaces in dairy and meat processing plants (Schlegelová *et al.* 2010).

More studies are needed concerning the prevalence of multi drug resistant (MDR) staphylococci in food processing environment, food handlers, and the products in order to obtain sufficient data for the correct risk assessment. Antimicrobial and especially genotype profiles of the isolated strains are useful tools for the epidemiological studies in the food manufacturing plants and the identification of the potential food contamination sources.

CONCLUSIONS

The presence of MDR *S. aureus* in RTE foods, such as salads, represents a potential threat for



Lanes 1–6: isolates from personnel's nasal cavities; lanes 7–12: isolates from salads; L – molecular weight DNA ladder

Figure 1. RAPD profiles of representative *Staphylococcus aureus* isolates

the acquisition of antimicrobial resistant genes by those who eat or handle those foods. However, since *S. aureus* isolates from handlers were different from those from salads, the application of Good Manufacturing and Hygiene Practices along the food chain seems to be a rule of thumb to prevent microbial spread and to eliminate the transfer of antimicrobial resistance genes through foods.

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